“Take My Bone Away?” Hypoxia and bone: A narrative review

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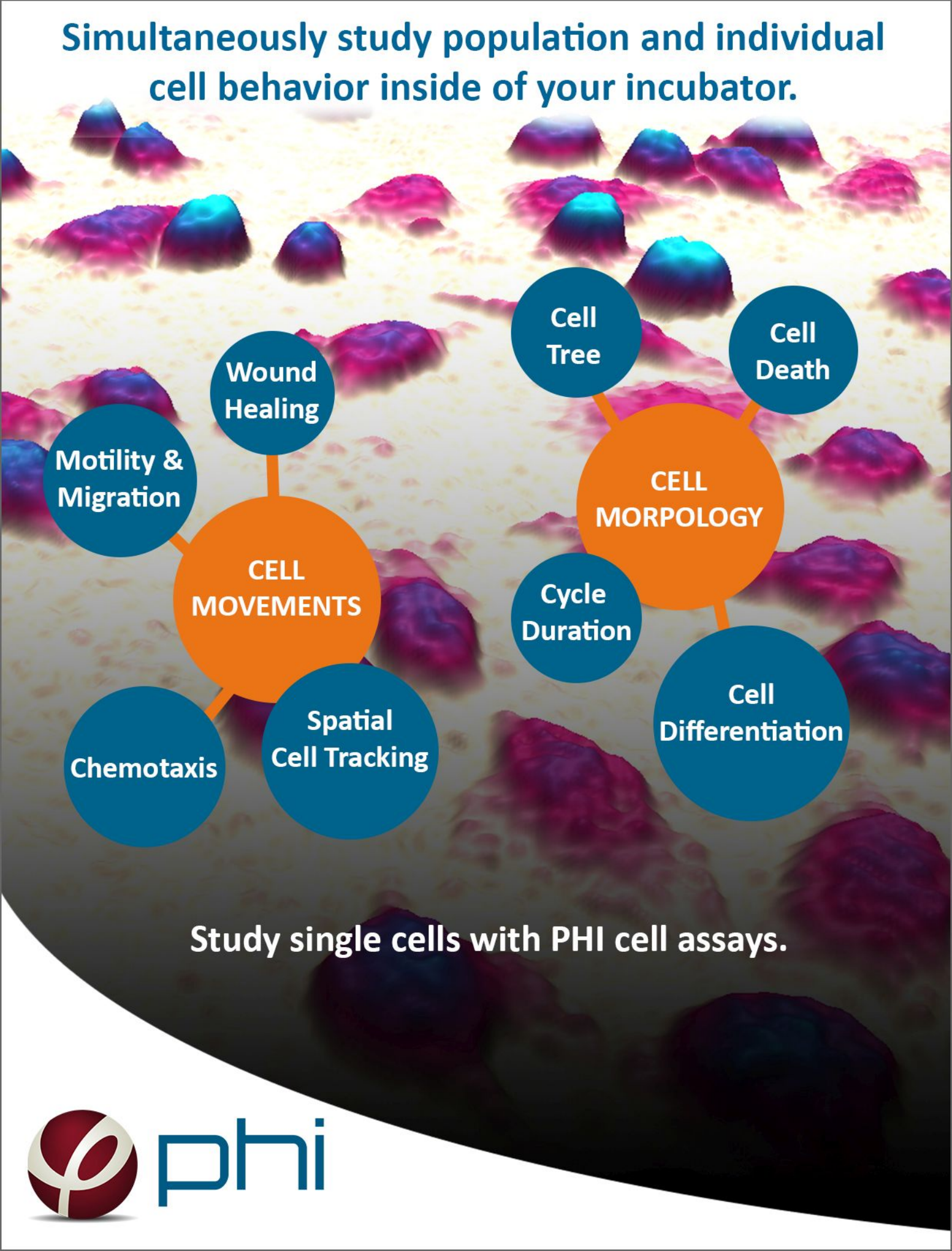

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MINI-REVIEW

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"Take My Bone Away?" Hypoxia and bone: A narrative review

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Abstract

To maintain normal cellular and physiological function, sufficient oxygen is required. Recently, evidence has suggested that hypoxia, either pathological or environmental, may influence bone health. It appears that bone cells are distinctly responsive to hypoxic stimuli; for better or worse, this is still yet to be elucidated. Hypoxia has been shown to offer potentially therapeutic effects for bone by inducing an osteogenic–angiogenic response, although, others have noted excessive osteoclastic bone resorption instead. Much evidence suggests that the hypoxic-inducible pathway is integral in mediating the changes in bone metabolism. Furthermore, many factors associated with hypoxia including changes in energy metabolism, acid–base balance and the increased generation of reactive oxygen species, are known to influence bone metabolism. This review aims to examine some of the putative mechanisms responsible for hypoxic-induced alterations of bone metabolism, with regard to osteoclasts and osteoblasts, both positive and negative.

KEYWORDS

bone metabolism, HIF, hypoxia, metabolism, osteoblast, osteoclast, oxygen

1 | INTRODUCTION

To maintain normal cellular and physiological function, sufficient oxygen is required. In the absence of sufficient oxygen, the body is required to make adjustments to prolong survival. This physiological hypoxia arises from reduced environmental oxygen content—as seen with ascending altitude—or pathological factors, such as reduced tissue perfusion (Grocott et al., 2009; Levy et al., 2008; Luks & Swenson, 2011). Recently, evidence has shown that hypoxia, either pathological or environmental, may influence bone health (Tanaka, Minowa, Satoh, & Koike, 1992; Tando et al., 2016; Terzi & Yılmaz, 2016; W. Wang et al., 2017). Associations have been drawn between hypoxic-related conditions, such as anaemia, sleep apnoea and chronic obstructive pulmonary disease and poor bone mineral density (BMD; Ramachandran, Mani, Gopal, & Rangasami, 2016; Terzi & Yılmaz, 2016; Valderrábano et al., 2017). Although these relationships offer little causative evidence, experimental research (mostly in animal models) has suggested that hypoxia directly affects bone health (Arnett, 2010; Basu et al., 2013; W. Wang et al., 2017). For example, mice exposed to simulated 6000 m altitude (~9.8% O_2) for 21 days were reported to have 33% less trabeculae bone volume when compared to controls (W. Wang et al., 2017). The structural changes observed in this exposure group significantly reduced histomorphometric measures of bone strength, indicating that hypoxic exposure may increase the risk of bone fracture (W. Wang et al., 2017). This review aims to examine some of the putative

Abbreviations: ALP, alkaline phosphatase; AMPK, 5′-adenosine monophosphate-activated protein kinase; COX, cyclooxygenase; EPO, erythropoietin; FGF23, fibroblast growth factor 23; FIH, factor-inhibiting HIF; FOX, forkhead box; GLUT, glucose transporter; H_2O_2, hydrogen peroxide; HIF, hypoxic-inducible factor; NFATc1, nuclear factor-activated T cells c1; OPG, osteoprotegerin; OVX, ovariectomised; PDH, pyruvate dehydrogenase; PHD, prolyl hydroxylase domain; PI3K, phosphoinositide 3-kinase; pVHL, von Hippel–Lindau protein; RANK, receptor activator of nuclear factor κB; RANKL, receptor activator of nuclear factor κB ligand; ROS, reactive oxygen species; Runx2, runt-related transcription factor 2; STAT, signal transducer and activator of transcription; TCA, tricarboxylic acid cycle; TRAP, tartrate-resistant acid phosphatase; VEGF, vascular endothelial growth factor.

mechanisms responsible for hypoxic-induced alterations of bone metabolism including: hypoxic pathway activation; metabolic alterations; reactive oxygen species generation; acidosis; and erythropoietin production.

1.1 | Overview of basic multicellular units (BMUs)

Normal bone metabolism is governed by a BMU. The BMU consists of osteoclasts resorbing bone; osteoblasts forming bone; and osteocytes regulating bone metabolism and sensing mechanical forces (Kular, Tickner, Chim, & Xu, 2012). While the intricacies of the BMU are beyond the scope of this review, it is important to note that the BMU operates in a well-orchestrated manner to correctly regulate bone turnover and that insufficiencies in the crosstalk and signalling processes can result in maladaptation (Kular et al., 2012; Raubenheimer, Miniggio, Lemmer, & van Heerden, 2017). Generally, bone turnover works in three distinct phases: (a) initiation; (b) reversal; and (c) termination. The initiation phase recruits osteoclast precursors, differentiates them to mature multinucleated osteoclasts and activates them for resorption. This initiation phase largely operates through the binding of receptor activator of nuclear factor-κB ligand (RANKL)- which is produced by osteoblasts, endothelial cells and T cells- to receptor activator of nuclear factor κB (RANK)- present on osteoclasts and their precursors (Asagiri & Takayanagi, 2007; Burgess et al., 1999; Lacey et al., 1998; Xu et al., 2000). The reversal phase involves the inhibition of osteoclastic activity and apoptosis while osteoblasts differentiate before entering the termination phase where bone formation occurs (Eriksen, Gundersen, Melsen, & Mosekilde, 1984; Eriksen, Melsen, & Mosekilde, 1984; Raggatt & Partridge, 2010). Regulation of the BMU is a complex interplay between cell signalling and endocrine influence (Kular et al., 2012). Interestingly, hypoxia is believed to stimulate/alter several factors capable of manipulating the BMU, such as, hypoxia-induced factors, energy metabolism, acidosis, reactive oxygen species (ROS) generation and erythropoietin (EPO) production (Arnett, 2010; Bartell et al., 2014; Hiram-Bab, Neumann, & Gabet, 2017).

1.2 | Overview of the cellular response to hypoxia

In the simplest form, hypoxia can be defined as the threshold whereby normal cellular function is limited by the oxygen concentration (Zepeda et al., 2013). Hypoxic stimuli, such as low ambient oxygen partial pressure/levels, poor oxygen diffusion and perfusion, may initiate a cellular hypoxic response. Although the earth's atmosphere consists of 20.9% oxygen, tissue oxygen typically ranges from 2% to 9% (Marenzana & Arnett, 2013; Reyes et al., 2012). Therefore, small changes in atmospheric oxygen can potentially lead to important relative changes in cellular oxygen, which in turn stimulate hypoxic pathways. The cellular response to hypoxia is largely mediated through heterodimeric transcription factors: the hypoxia-inducible factors (HIFs; Déry, Michaud, & Richard, 2005). Each HIF consists of a HIF-α subunit and a constitutively expressed beta subunit, HIF-β. In normoxia (>~5% O_2), HIF-α is post-translationally hydroxylated by the enzymes prolyl hydroxylase domains (PHDs) 1–3 for subsequent interaction with the von Hippel–Lindau protein (pVHL) for poly-ubiquitination and proteasomal degradation. Furthermore, factor-inhibiting hypoxia (FIH) hydroxylates the HIFs asparagine residue, inhibiting the ability of HIF-α to recruit its transcriptional capability (Bruick, 2001; Epstein et al., 2001; Knowles, 2015a). Whereas under hypoxia (<~5% O_2), PHD activity is reduced, leading to HIF-α accumulation and translocation to the nucleus. HIF-α then dimerises with HIF-β and binds to the hypoxia-response element of HIF target genes, initiating transcription of more than two hundred genes (Déry et al., 2005; Kaluz, Kaluzová, & Stanbridge, 2008; Semenza, 2003; see Figure 1). The activation of HIFs result in the stimulation of several physiological pathways such as: angiogenesis; pH regulation; cellular apoptosis; and glycolysis, all of which are imperative for survival in hypoxic environments (Hu, Wang, Chodosh, Keith, & Simon, 2003; J.-W. Lee, Bae, Jeong, Kim, & Kim, 2004).

2 | HYPOXIA AND BONE

The level of oxygen reaching bone tissue is thought to be around 6.6–8.6% O_2, as measured in bone aspirates (Harrison, Rameshwar, Chang, Bandari, & Persis, 2002). Considering this, it is entirely conceivable that exposure to small changes in either inspired O_2 or O_2 delivery may influence cell homoeostasis, namely by stimulation of HIF pathways (Arnett, 2010; Harrison et al., 2002; Marenzana & Arnett, 2013). Although hypoxia is a stimulus for HIF stabilisation, we consider the effects of HIF-1 and HIF-2 separately to the overall effect of physiological hypoxia.

2.1 | Osteoblast

Utting et al. (2006) were among the first to note decreases in osteoblast bone formation when exposed to low-oxygen environments. Following in vitro osteoblast exposure to 2% O_2, bone formation decreased 10-fold and was almost ablated in 0.2% O_2. Hypoxia was noted to delay both osteoblast growth and differentiation, limiting overall bone formation (Utting et al., 2006). Similarly, short-term hypoxic exposure in ovariectomised (OVX) rats has been shown to supress osteoblastogenesis further than OVX alone (Xian et al., 2016). Reductions in osteoblastogenesis have been attributed to reduced Runx2 expression and activity in hypoxia, subsequently reducing multipotent mesenchymal cell differentiation to immature osteoblasts (Komori, 2010; Ontiveros, Irwin, Wiseman, & McCabe, 2004; Park, Park, Kim, Park, & Baek, 2002; Salim, Nacamuli, Morgan, Giaccia, & Longaker, 2004). Several in vitro studies have also noted reductions in osteoblastogenesis through inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathways that are normally involved in the antiapoptotic and survival function in cells (H. P. Ma et al., 2014; Zou et al., 2014).

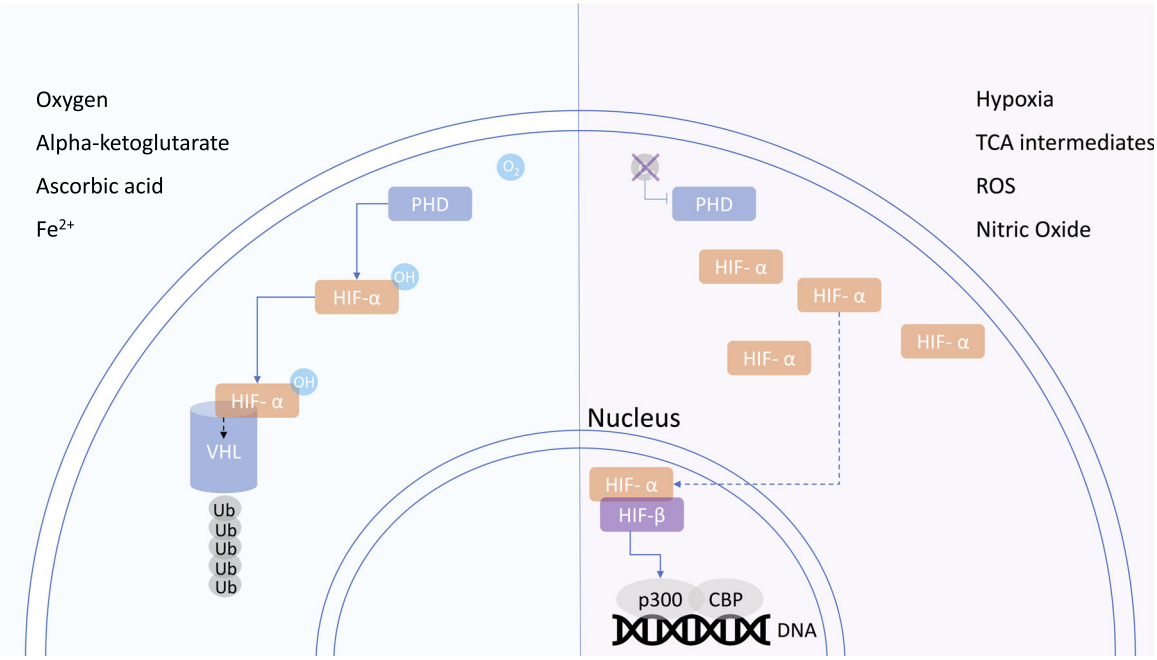

FIGURE 1 HIF pathway activation. The left-hand side shows the moderators of rapid degradation of HIF-α through posttranslational hydroxylation by the enzymes PHD 1-3 and poly-ubiquitination and proteasomal degradation via the VHL protein. The right-hand side shows that under hypoxia and other mediators PHD activity is inhibited, leading to HIF-α accumulation and translocation to the nucleus. HIF-α then dimerises with HIF-β and binds to transcriptional co-activators p300/CBP to elicit activation of the hypoxia-response element for transcription of hypoxic genes. HIF, hypoxic-inducible factor; PHD, prolyl hydroxylase domain; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle; VHL, von Hippel–Lindau

In addition to reduced osteoblastogenesis, osteoblast matrix mineralisation is also inhibited in hypoxia, as a result of reduced expression and activity of alkaline phosphatase (ALP; Utting et al., 2006). Hypoxic-induced inhibition of osteoblast function may also be the result of reduced PHD and lysyl oxidase enzyme activity (Arnett, 2010; Utting et al., 2006). These oxygen-dependent enzymes are required for posttranslational modification of collagen; however, they are reduced during hypoxia, resulting in impaired collagen cross-linking (Myllyharju, 2003; Utting et al., 2006). It may be the combination of reduced matrix mineralisation and collagen cross-linking that severely limits osteoblastic function (Utting et al., 2006). Furthermore, osteoblasts have also been shown to release adenosine triphosphate (ATP) in response to hypoxia, which concurrently inhibits bone formation and stimulates osteoclasts (Morrison, Turin, King, Burnstock, & Arnett, 1998; Orriss et al., 2009). Generally, hypoxic exposures inhibit osteoblast activation and osteoblast numbers.

2.2 | Osteoclasts

The effect of hypoxia on osteoclasts appears more definitive than that of osteoblasts. Exposure to 2% O_2 has been shown to stimulate increases in osteoclast number and activity (Arnett et al., 2003; Knowles & Athanasou, 2009; Muzylak, Price, & Horton, 2006; Utting, Flanagan, Brandao-Burch, Orriss, & Arnett, 2010). In murine bone marrow cultures, osteoclast activity has been reported to increase 21-fold following exposure to 2% O_2 (Arnett et al., 2003). Similarly, 2% O_2 increased resorption pit formation 10-fold in osteoclasts differentiated from human peripheral blood mononuclear cells (Utting et al., 2010). It first was noted that hypoxia maximally stimulated osteoclastogenesis at 2% O_2, resulting in a four-fold increase in osteoclast number (Arnett et al., 2003). Others have noted similar increases in osteoclast numbers when exposed to hypoxia (Muzylak et al., 2006; Utting et al., 2010).

It is important to consider the hypoxic protocols used in such studies as constant hypoxic exposure results in inhibition of osteoclast formation and activity, due to extensive cell death (Knowles & Athanasou, 2009; Z. Ma et al., 2019). However, reoxygenation every 2–3 days has been shown to promote osteoclastogenesis (Knowles & Athanasou, 2009). Clearly, osteoclast sensitivity to O_2 is partially responsible for the need to re-oxygenate for continued differentiation (Knowles, 2015a). The increase in osteoclast numbers following intermittent hypoxic exposure may also be related to increases in reactive oxygen species which may account for the increased differentiation is some studies (ROS: discussed later). Mechanistically, hypoxia has been shown to supress the RANKL decoy receptor, osteoprotegrin (OPG), preventing RANK-induced osteoclast formation and activity (Shirakura et al., 2010; Xian et al., 2016), although this has not been consistently demonstrated (Hulley et al., 2017; Kang et al., 2017; S. Y. Lee et al., 2019; Merceron et al., 2019; Shao et al., 2015; Wu et al., 2015). It is difficult to ascertain this inconsistency, but it may be explained by experimental variations of

HIF-induction (hypoxia or gene editing), cell lines or single cell line work opposed to coculture or in vivo. Shirakura et al. (2010) demonstrated that HIF-1α knockdown could only significantly increase OPG under hypoxic conditions and not normoxic. Hypoxia's downstream effects may mediate OPG decreases and that HIF-induction alone may increase OPG or not at all. Although, it has also been suggested that osteoclasts and osteoblasts have differing sensitivities to HIF that is osteoclasts are more sensitive to HIF inhibition whereas osteoblasts appear more sensitive to HIF activation (Knowles, 2015b). While hypoxia appears to inhibit osteoblast function and number, it can stimulate these in osteoclasts, although constant exposure may negate this. Some of the putative mechanisms are alluded to above but we herein examine the primary suspects in mediating the generally reported negative effect of hypoxia on bone below.

3 | HIF-1α: FRIEND OR FOE?

3.1 | Osteoblasts

Early research on hypoxia and osteoblasts provided insightful results: Y. Wang et al. (2007) first reported that hypoxic/HIF stimulation led to increased bone formation in mice via HIF-1α-dependent induction of the angiogenic factor, vascular endothelial growth factor (VEGF). Subsequent research noted the importance of HIF-1α signalling in skeletal development, terming it osteogenic–angiogenic coupling (C. Wan et al., 2010; Wu et al., 2015). It was suggested that hypoxic exposure induces HIF-1α transcription of VEGF improving local vascularisation and, in turn, increasing activation of target genes, progenitor cells and nutrients enhancing bone formation (Schipani, Maes, Carmeliet, & Semenza, 2009; Y. Wang et al., 2007). Using PHD inhibition, X. Liu et al. (2014) demonstrated improved structural measures of bone in OVX mice (X. Liu et al., 2014). This was likely the result of the increased number of osteoblasts in mice overexpressing HIF-1α when compared to controls (Y. Wang et al., 2007). Such findings led the same authors to hypothesise that HIF-1α stimulation may augment skeletal repair. Using a skeletal repair model, mice overexpressing HIF-1α demonstrated increased vascularity and bone formation, whereas mice lacking HIF-1α displayed significantly poorer repair (C. Wan et al., 2010). The mechanism responsible for the HIF-1α driven bone formation is thought to be mediated through the upregulation of glycolytic activity, subsequently increasing osteoblast activity (Regan et al., 2014; see Section 9.2). This tentative evidence highlights a putative role for augmented HIF-1α stimulation in the treatment of poor bone health, but given the lack of human studies, such approaches should be viewed with caution. Finally, it should be noted that the osteoanabolic response is attributed to HIF-1α stimulation alone and may not prevail in hypoxia, due to the multifaceted nature of reduced oxygen content or delivery. Utting et al. (2006) has suggested that this discrepancy in bone cell responses may be due to in vivo systems eventually reaching sufficient oxygen for increased bone formation via osteogenic-angiogenesis. Whereas, in vitro, VEGF would seemingly be unable to yield an anabolic response in the absence of oxygen. The disparity in response remains poorly understood and future work should aim to further explore this variability.

3.2 | Osteoclasts

While osteoblasts appear capable of yielding an osteoanabolic response to HIF-1α stabilisation, osteoclasts appear to provide an antagonistic response. The hypoxic-induced resorption of osteoclasts has been reported to be HIF-1α dependent (Knowles & Athanasou, 2009). This was first noted when HIF-1α siRNA completely ablated the hypoxic increase in resorption (Knowles & Athanasou, 2009); although, it has since been suggested that the hypoxic-induction enzyme, PHD2 and HIF-1α play direct roles in hypoxic enhanced resorption (Hulley et al., 2017). Hulley et al. (2017) found that heterozygous depletion of PHD2 in bone marrow cells increased expression of pro-resorptive genes, resulting in 3.7-fold higher resorption when compared to wild type. In vivo deletion of PHD2 echoed in vitro observations: increased resorption and subsequently reduced BV/TV, trabecular number and increased trabecular spacing. The manner in which HIF-1α affects osteoclast function still requires clarification but appears to be related to the stimulation of pro-resorptive genes and glycolytic activity stimulating resorption (discussed later; Knowles, 2015a). Nonetheless, it would seem evident that the hypoxic-induced increase in osteoclast activity is, in part, HIF-1α mediated.

While the HIF-1α-induced increase in osteoclast activity is well documented, the role of HIF-1α in osteoclastogenesis is less clear. Several reports note increased differentiation following HIF-1α stimulation, while others have noted decreases (Bozec et al., 2008; Leger et al., 2010; Miyauchi et al., 2013). More recently, HIF-1α siRNA was shown to accelerate osteoclast cell fusion while HIF-1α induction moderately inhibited differentiation (Hulley et al., 2017). Intriguingly, HIF-1α stabilisation with hypoxic mimics, cobalt and L-mimosine, significantly reduced osteoclast differentiation and subsequently resorption, whereas hypoxia did not. Hypoxic-induction of HIF-1α caused a nonsignificant decrease in osteoclast numbers, which the authors attribute to the longer reoxygenation times compared to other studies (Arnett et al., 2003; Hulley et al., 2017; Knowles & Athanasou, 2009; Muzylak et al., 2006; Utting et al., 2010). Hypoxia did however increase resorption early on during differentiation, significantly increasing the final resorption capacity (Hulley et al., 2017). The discrepancy in differentiation between hypoxic mimics and hypoxia may be explained by the fact that cobalt and L-mimosine are not PHD specific inhibitors, whereas hypoxia is. Considering PHD2 is partly responsible for the hypoxic-induced increase in osteoclast activity, it is important to consider whether there is a role for PHD in differentiation. In vivo deletion of PHD2 had no effect on the number of TRAP-positive osteoclasts (Hulley et al., 2017). Homozygous knockdown of PHD3 mirrors the HIF1-α siRNA response, accelerating osteoclast formation, which is associated with increased expression of the differentiation marker nuclear

factor-activated T cells c1 (Nfatc1; Hulley et al., 2017). Yet, this did not affect the final number of osteoclasts formed. One explanation may be that PHD3 depletion stimulates PHD2 expression to inhibit HIF-1α stabilisation and negate the catabolic effects, but this remains speculative. It is possible that the hypoxic-induced increase in osteoclast number is the result of osteoblast-osteoclast crosstalk (Shirakura et al., 2010; Xian et al., 2016), although studies examining osteoclasts alone were able to demonstrate increased differentiation, suggesting mediation by downstream effects (Arnett et al., 2003; Brandao-Burch, Meghji, & Arnett, 2003; Kato & Matsushita, 2014; Knowles, 2019; Utting et al., 2010).

4 | HIF-2: TOO GOOD TO BE TRUE?

The role of HIF-1 in bone metabolism appears to be well documented when compared to HIF-2. Though HIF transcription factors share similarities and overlapping roles, they can have opposing actions. Here we discuss HIF-2's emerging role in bone metabolism.

4.1 | Osteoblasts

Initial research identified that loss of HIF-2 in both osteoprogenitors and osteoblasts did not impact osteoblast activity or number (Shomento et al., 2010; Wu et al., 2015). However, it has recently been reported that HIF-2 is a negative regulator of osteoblastogenesis (S. Y. Lee et al., 2019; Merceron et al., 2019). Deficiency of HIF-2 increases bone mass through promoting *osteoblast* differentiation and inhibiting *osteoclast* differentiation (Merceron et al., 2019). HIF-2's impairment of osteoblastogenesis is partly regulated through Sox9—a negative regulator of osteoblast differentiation (Merceron et al., 2019; Zhou et al., 2006). Sox9 appears to limit differentiation through reductions in expression of the important osteoblast differentiation mediators, Runx2 and Sp7. Although previous research has suggested that HIF-induction of VEGF promotes osteoangiogensis, Merceron et al. (2019) demonstrated that despite increased expression of VEGFA mRNA in PRX-HIF2dPA$^{f/+}$ mice, the osteoangiogenic response was inhibited. Stabilisation of HIF-2 appears to supersede the anabolic actions of VEGFA and inhibit bone formation and osteoblastogenesis (Merceron et al., 2019).

Similarly, HIF-2α deficiency is shown to promote osteoblast differentiation and increase bone formation in mice (S. Y. Lee et al., 2019). S. Y. Lee et al. (2019) suggest that HIF-2α mediates its inhibitory actions on osteoblasts through the target gene Twist2. Twist2 inhibits RUNX2 and osteocalcin expression, resulting in reduced osteoblast mineralisation and bone mass (S. Y. Lee et al., 2019). TWIST is also a downstream target of hypoxia and HIF-1α stabilisation, which inhibits the expression of type 1 RUNX2. Such decreases in RUNX2 further inhibit expression of BMP-2, type 2 RUNX2 and subsequently osteoblast mineralisation (Yang et al., 2011). Therefore, in hypoxic microenvironments where HIF stabilisation occurs more ubiquitously, expression of TWIST may be greater and could explain reductions in RUNX2 in hypoxic environments (Komori, 2010; Ontiveros et al., 2004; Park et al., 2002; Salim et al., 2004).

4.2 | Osteoclasts

HIF-2α deficiency appears to influence osteoclasts through both primary and secondary mechanisms. HIF-2α overexpression in osteoclasts with M-CSF and RANKL treatment enhanced differentiation, as evidenced by the increase in number of TRAP-positive cells (S. Y. Lee et al., 2019). The overexpression of HIF-2α resulted in large osteoclast formation with large cytoplasmic compartments, suggesting that HIF-2α may also stimulate osteoclast maturation, similar to HIF-1α (Hulley et al., 2017; S. Y. Lee et al., 2019). The increased expression of osteoclast-fusion related genes, during M-CSF and RANKL-induced osteoclastogenesis, supports the notion that HIF-2α is capable of stimulating and accelerating osteoclastogenesis (S. Y. Lee et al., 2019). Interestingly, inhibition of HIF-2α reduced the RANKL-mediated differentiation of osteoclasts as evidenced by the reduced number of nuclei and the expression of osteoclast-related genes in a dose-dependent manner. The direct molecular mechanism of HIF-2α mediated osteoclastogenesis may be due to the upregulation of the target gene TRAF6 (S. Y. Lee et al., 2019). TRAF6 is an adapter of RANK, leads to Nfatc1 activation and promotes osteoclastogenesis (Gohda et al., 2005; Kanemoto et al., 2015; S. Y. Lee et al., 2019). TRAF6 expression increased during RANKL-mediated osteoclast differentiation but when combined with HIF-2α overexpression, its expression was enhanced. Similarly, when HIF-2α was inhibited so was TRAF6 (S. Y. Lee et al., 2019). Inhibition of TRAF6 blocked the HIF-2α induced increase in osteoclast differentiation and formation (S. Y. Lee et al., 2019).

HIF-2 stabilisation appears to regulate aspects of differentiation and osteoclastogenesis but not osteoclastic resorption (Knowles, 2015b). Silencing of HIF-2α in human monocyte-derived osteoclasts had no effect on the hypoxic-induced resorption when cells were exposed to hypoxia (Knowles & Athanasou, 2009; Knowles, Cleton-Jansen, Korsching, & Athanasou, 2010). Others have noted that HIF-2α is capable of increasing mineral resorption, evidenced by the increase in osteoclast activity genes: Trap, Ctsk and Nfatc1 (S. Y. Lee et al., 2019). However, this may also be the result of the increased osteoclast number seen with HIF-2α stabilisation.

It has been suggested that HIF-2α is an important mediator of osteoblast-osteoclast crosstalk. Osteoclast-specific loss of HIF-2α increases bone mass via affecting solely osteoclasts, whereas osteoblast-specific loss of HIF-2α increases bone mass via affecting osteoblasts and osteoclasts (S. Y. Lee et al., 2019). HIF-2 may directly bind to the RANKL promoter to increase osteoclast differentiation (S. Y. Lee et al., 2019; Ryu et al., 2014). However, it has also been suggested that the HIF target gene, OPG and its intermediary interleukin 33, may be responsible for the osteoblast-mediated inhibition of osteoclastogenesis (Kang et al., 2017; Merceron et al., 2019; Shao et al., 2015; Wu et al., 2015). It seems plausible that part

of the osteoclastogenic response to hypoxia may be mediated through independent cell action but also osteoblast-osteoclast crosstalk.

5 | BRIEF SUMMARY: HYPOXIA, HIF-1 AND HIF-2

True hypoxia—a reduction in oxygen availability—results in the concomitant stabilisation of HIFs. Although similar, HIF-1 and HIF-2 have different oxygen sensitivities (Greer, Metcalf, Wang, & Ohh, 2012; Patel & Simon, 2008). Intuitively, one would expect reduced oxygen availability and HIF stabilisation to have similar effects; however, this is not the case and antagonistic relationships have been demonstrated in other tissues (Hu et al., 2003; Maxwell et al., 1999; Raval et al., 2005; V. Wang, Davis, Haque, Huang, & Yarchoan, 2005). Therefore, any hypoxic exposure should be considered carefully, as the level and duration of hypoxia may elicit differential HIF stabilisations. This may account for why some hypoxic exposures elicit potentially favourable changes whereas others show potentially detrimental effects.

In Figure 2, we summarise the hypoxic signalling effects on both osteoclasts and osteoblasts. Hypoxia generally stimulates osteoclasts and inhibits osteoblasts. While HIF-1a stimulates both osteoblasts and osteoclasts, HIF-2 stimulates osteoclasts but inhibits osteoblasts. HIF-1 and HIF-2 both stimulate angiogenesis but only HIF-1 is capable of yielding an anabolic response as HIF-2 inhibits osteoblast differentiation and proliferation (Shomento et al., 2010). Therefore, in hypoxia where both HIF-1 and HIF-2 stabilisation occurs, HIF-2 appears to limit the anabolic response. Although it could be argued that the increase in VEGF during HIF-2 stabilisation was insufficient to elicit the anabolic response (S. Y. Lee et al., 2019). However, it has been suggested that HIF may exert differential effects depending on age and bone cycle (modelling vs. remodelling). Research examining bone *modelling* in young mice has found that HIF-2 has no significant effect on bone mass, albeit this may have been the result of only a

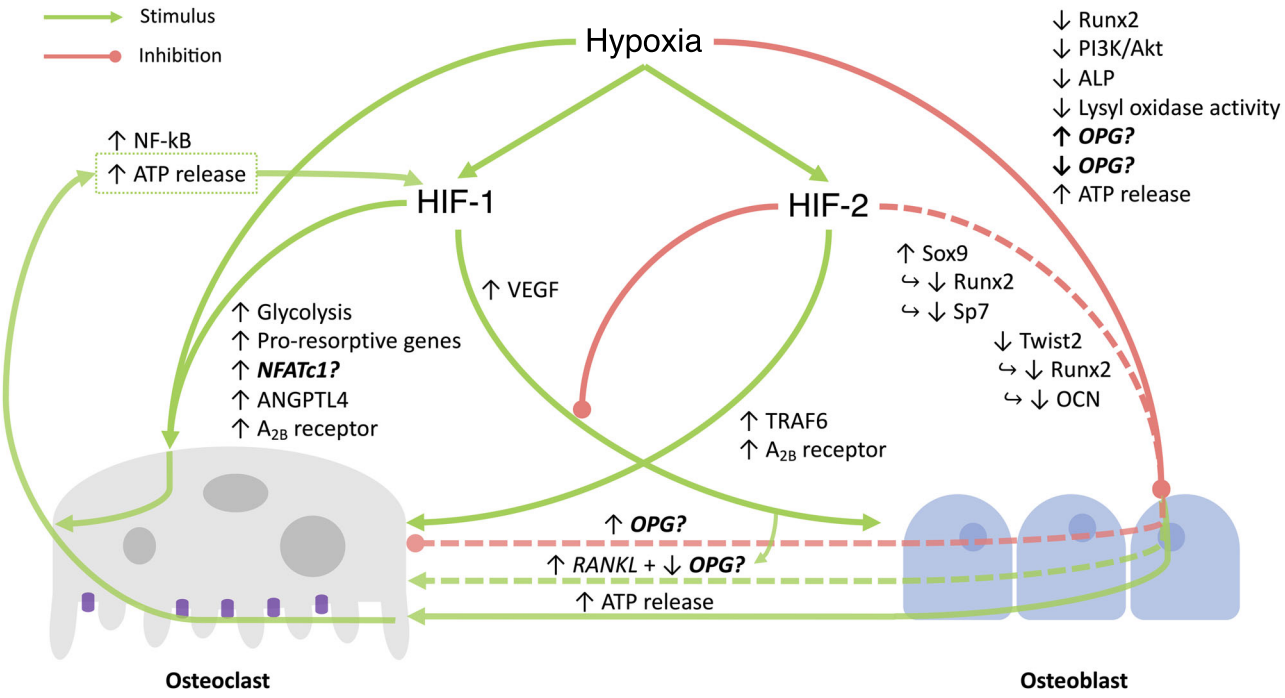

FIGURE 2 Summary of hypoxia and HIF regulation on osteoclasts and osteoblasts. Hypoxia stimulates osteoclast activity and number while also inhibiting osteoblast activity and number through numerous mechanisms. Hypoxia induces HIF-1 and HIF-2 stabilisation, which exert differential effects on osteoclasts and osteoblasts. HIF-1 stimulates osteoclast activity through the upregulation of pro-resorptive and glycolytic genes, and ATP release which subsequently invokes further ATP release and HIF-1α stabilisation. HIF-1 also stimulates increases in VEGF which stimulates osteo-angiogenesis by increasing osteoblast bone formation and number. HIF-2 may negatively regulate of osteoblast differentiation, mediated by increases in Sox9 and decreases in Twist2 and subsequent decreases in Runx2, Sp7 and osteocalcin. HIF-2 may also inhibit the osteoangiogenic response mediated by HIF-1, but this remains speculative. HIF-2 exerts both a direct effect and indirect effect on osteoclasts. HIF-2 stimulates increases in TRAF6 which stimulate RANKL-induced osteoclastogenesis but has also been shown to increase and decrease OPG and RANKL. Further research is needed to fully understand bone cell crosstalk during hypoxia. Finally, osteoblasts release ATP in response to hypoxia which, as previously mentioned, stimulate osteoclast activity. The purinergic signalling appears to be cyclic, whereby hypoxia releases ATP from both cells, which drives further HIF-1α stabilisation and subsequent increased ATP release (discussed further below). Key: green lines indicate stimulation; red lines indicate inhibition; dashed lines indicate HIF-2 mediated; and contrasting evidence is bold followed by a question mark. HIF, hypoxic-inducible factor; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κB ligand; VEGF, vascular endothelial growth factor

modest increase in VEGF expression (S. Y. Lee et al., 2019). Whereas, during bone *remodelling*, HIF-2 is crucial in regulating osteoblast differentiation (S. Y. Lee et al., 2019). HIF-1 is clearly the crucial anabolic response element with regard to the osteogenesis-angiogenesis phenomenon but HIF-2 may contribute to remodelling through alteration of osteoblast and osteoclast key markers (S. Y. Lee et al., 2019).

The regulation of bone metabolism through HIF is admittedly complex and comparisons must be drawn carefully especially in different physiological/pathological contexts. For example, consider HIF stabilisation in OVX-induced osteoporosis: deficiency of oestrogen increases cytokine expression, which subsequently may affect the level of HIF stabilisation and negate HIF-induced actions.

5.1 | Stimulating hypoxia

While HIF are the hypoxic regulators, they do not necessarily require hypoxia to be stimulated. Several factors stimulate the HIF-1α pathway including: hypoxic mimicking agents such as cobalt or L-mimosine; gene therapy; and iron chelators (Drager, Harvey, & Barralet, 2015). Interestingly, bone cells appear to respond differently to each of these stimuli. While stimulation of the HIF-1α pathway with cobalt or L-mimosine has been shown to stimulate bone resorption, it has also been shown to reduce the final number of osteoclasts when compared to hypoxia (Hulley et al., 2017). This direct comparison may offer some explanation of the variance seen in bone cell responses to HIF-1α stabilisation. Similarly, iron chelators produce varying outcomes depending on cell iron specificity and membrane permeability (Cho et al., 2013). The nonspecific iron chelator, desferoxamine (DFO), is shown to produce greater FIH and PHD enzyme inhibition when compared to the specific Fe^{2+} iron chelators, 1, 10-phenanthroline and dipyridyl (Cho et al., 2013). Cho et al. (2013) suggests that such discrepancies may be the result of cellular Fe^{2+} maintaining FIH-1 while DFO inhibits PHD, possibly explaining why HIF-1α and EPO expression were significantly higher in specific Fe^{2+} iron chelators.

More recently, RNA sequencing has demonstrated that PHD and VHL inhibitors produce different transcriptional responses to hypoxia (Frost, Ciulli, & Rocha, 2019). While the PHD inhibitor, IOX2 and the VHL inhibitor, VH032 mimic the hypoxic response, hypoxia induces a broader transcriptional response. A common expression of 306 genes was observed between hypoxia, PHD and VHL inhibitors, however, hypoxia induced a significantly greater gene repression response. Hypoxic transcriptional repression has been associated with several mechanisms; the transcriptional regulatory protein, SIN3A, has been attributed to 75% of hypoxia-repressed genes (Batie, del Peso, & Rocha, 2018; Tiana et al., 2018). Furthermore, knockdown of SIN3A inhibited approximately 47% of upregulated hypoxic genes (Tiana et al., 2018). The complex transcriptional regulation seen under hypoxia may offer some insight into the varying responses in bone cells.

Some of the equivocal data may be a function of the level of "hypoxia" used in the highlighted studies. However, as previously mentioned, bone O_2 content rests between 6.6% and 8.6% (Harrison et al., 2002). Therefore, it is possible this may not have provoked a meaningful effect. Others have reported significant increases in resorption and differentiation using 2% O_2, suggesting a possible hypoxic threshold (Arnett et al., 2003; Knowles & Athanasou, 2009; Muzylak et al., 2006; Utting et al., 2010). As such, the wide degree of variance stimulating cellular "hypoxia" makes it difficult to ascertain the true bone cell response. Whereas the activation of the HIF transcription factor appears to be integral to the bone response, limiting or inhibiting different components of the hypoxia signalling pathway appears to exert differential effects. Further research is needed to assess the intricacies of hypoxic mimicking agents and gene editing to note whether these truly represent the physiological stress of hypoxia.

6 | METABOLISM AND BONE

Activation of the HIF pathway is important in moderating many cellular responses and adaptations to hypoxic stress. To operate effectively in hypoxic environments, a shift from aerobic to anaerobic metabolism is required. HIF signalling typically increases energy supply and demand via glycolysis, maintaining normal cellular function in low-oxygen environments (Majmundar, Wong, & Simon, 2010). However, in bone, metabolic functionality differs in several distinct ways to most tissues (discussed below), which may offer insight for the effects exerted by hypoxia (Knowles, 2015b).

6.1 | Osteoclasts

Osteoclasts are inherently glycolytic cells often characterised by their motility, numerous mitochondria and high expression of citric acid cycle and oxidative phosphorylation enzymes (Lemma et al., 2016). This is evident during normal monocyte-osteoclast differentiation where there is a steady but consistent increase in glucose consumption (Indo et al., 2013; J. M. Kim et al., 2007; Lemma et al., 2016). Osteoclast glucose dependence exceeds differentiation and manifests as the primary energy source for bone resorption (Indo et al., 2013; Williams et al., 1997). Considering hypoxia is a primary regulator of metabolic shift and glucose metabolism (Nakazawa, Keith, & Simon, 2016) and that osteoclasts demonstrate a high dependence on glycolysis, it is interesting to consider how hypoxia influences osteoclast function.

It is plausible to suggest that the high glycolytic rate of osteoclasts is accentuated by hypoxia. For example, HIF stimulation leads to the upregulation of glucose transporters and enzymatic genes (Morten, Badder, & Knowles, 2013), subsequently increasing osteoclast glucose consumption (Cramer et al., 2003; Larsen, Falany, Ponomareva, Wang, & Williams, 2002). The increased exposure of glucose subsequently leads to transcription of A-subunit of vacuolar H^+ ATPase: the primary mechanism involved in acidifying the bone matrix (Larsen et al., 2002). A-subunit of vacuolar H^+ ATPase primarily relies on glucose to produce and secrete hydrochloric acid

into the resorption lacunae (Cappariello, Maurizi, Veeriah, & Teti, 2014). This is further supported as insufficient glucose reduces intracellular ATP and subsequently decreases bone resorption, as observed with glucose inhibitors (Cai et al., 2018; El Hajj Dib et al., 2006; B. Li & Yu, 2003; Morten et al., 2013). Henceforth, the synergistic relationship between hypoxia and glucose consumption may contribute to the increased resorption seen upon exposure.

To maintain the increased demand of glucose consumption under hypoxic conditions, osteoclasts make several interesting metabolic adjustments that deviate from the traditional HIF metabolic shift. Osteoclasts exposed to hypoxia exhibit increased levels of ATP, mitochondrial reductase activity and consume almost maximal levels of O_2 through the electron transport chain (ETC; Morten et al., 2013). Knowles (2015a, 2015b) suggests that osteoclasts undergo a form of "selective utilisation" of the typical HIF-mediated metabolic shift. For example, in hypoxic osteoclasts, pyruvate dehydrogenase (PDH) activity is not inhibited; BNIP3 production is not stimulated; and AMP-activated protein kinase (AMPK) phosphorylation is inhibited (Morten et al., 2013). Intracellular ratios of ATP:ADP or ATP:AMP may support AMPK de-phosphorylation and subsequent inactivation as a result of the high ATP production seen in osteoclasts (Knowles, 2015b).

More recently, purinergic signalling has been implicated as a potential mediator for hypoxic-induced bone resorption (Knowles, 2019). Following hypoxic exposure, osteoblasts and osteoclasts secrete adenosine (Knowles, 2019; Strazzulla & Cronstein, 2016). Furthermore, the P1 adenosine receptor, A_{2B}, is also hypoxic-inducible (Knowles et al., 2010) and thus presents a hypoxic-specific purinergic signalling pathway. Inhibition of the A_{2B} receptor during hypoxic exposure is shown to prevent hypoxic-induced bone resorption, somewhat through glycolytic and mitochondrial reductase attenuation (Knowles, 2019). Under hypoxic conditions, HIF stabilisation increases A_{2B} transcription and subsequent signalling due to both osteoblasts and osteoclast secretion of adenosine (Knowles, 2019). Increased A_{2B} activation further stimulates HIF-1α via an intracellular feedback loop leading to increased glycolysis and mitochondrial reductase activity, which as previously discussed, are intrinsically linked to osteoclast resorption (Knowles, 2019). Interestingly, A_{2B} inhibition in normoxia has no effect on resorption levels. It appears the A_{2B} receptor is hypoxic specific in this instance, where there is sufficient extracellular adenosine. Overall, hypoxic osteoclasts deviate from the classical metabolic shift via PDH activity, which results in significant mitochondrial metabolic flux and subsequent accumulation of mitochondrial ROS (Dandajena, Ihnat, Disch, Thorpe, & Currier, 2012; Srinivasan & Avadhani, 2007; Srinivasan et al., 2010). It appears plausible that hypoxia stimulates further glucose metabolism, which may be responsible for the increased number and activation of hypoxic osteoclasts.

6.2 | Osteoblasts

Similar to osteoclasts, osteoblasts are characterised with many mitochondria (Klein, Gal, Hartshtark, & Segal, 1993; Komarova, Ataullakhanov, & Globus, 2000; Passi-Even, Gazit, & Bab, 1993). However, unlike osteoclasts, osteoblasts mainly metabolise glucose into lactate, even in the presence of sufficient oxygen (Esen & Long, 2014). This 'aerobic glycolysis' prevails despite osteoblasts exhibiting active oxidative phosphorylation (Klein et al., 1993; Komarova et al., 2000; Passi-Even et al., 1993). HIF-1α stabilisation is seen to stimulate the glycolytic rate and enzymes in vivo (Regan et al., 2014). Interestingly, further research has identified that parathyroid hormone can stimulate an anabolic response in osteoblasts, achieved by increasing aerobic glycolysis indirectly via transcriptional induction of insulin insulin-like growth factor 1 (IGF-1). Subsequent induction of mTORC2 elevates numerous glycolytic enzymes and increases bone formation (Esen, Lee, Wice, & Long, 2015). Reducing aerobic glycolysis with dichloroacetate allows for greater pyruvate entry to the citric acid cycle that is subsequently shown to inhibit bone formation. This is supported in HIF-1α overexpression models (Regan et al., 2014), suggesting that osteoblasts are to some degree regulated through their metabolic pathways which appears to be the case when exposed to environmental hypoxia (Utting et al., 2006; W. Wang et al., 2017). The HIF-1α target gene, glucose uptake transporter 1 (GLUT1), has been highlighted to play a critical feed-forward role in osteoblast differentiation, whereby Runx2 is unable to successfully differentiate osteoblasts in its absence (Wei et al., 2015). Increases in glycolytic metabolism appear to be linked to positive osteoblast function, once again demonstrating the antagonistic relationship between osteoblasts and osteoclasts.

7 | REACTIVE OXYGEN SPECIES (ROS) AND BONE

7.1 | Osteoclasts

Hypoxia is known to stimulate many biochemical changes including increases in ROS (Dosek, Ohno, Acs, Taylor, & Radak, 2007; Moller, Loft, Lundby, & Olsen, 2001). ROS are reactive molecules containing oxygen and are produced during normal metabolism. However, excessive production of ROS can induce an imbalance between pro- oxidants and antioxidants resulting in oxidative stress and subsequent oxidative damage (Thannickal & Fanburg, 2000). ROS are ordinarily involved in bone resorption, but elevations in ROS and oxidative stress have been suggested to negatively affect bone metabolism (Wauquier, Leotoing, Coxam, Guicheux, & Wittrant, 2009). During monocyte-osteoclast differentiation, RANKL signalling requires ROS to serve as second messengers in signalling pathways that induce expression of NFATc1 (Callaway & Jiang, 2015; M. S. Kim et al., 2010; Nakashima & Takayanagi, 2011). Cathepsin K-mediated degradation of TRAP, a bone resorption process, activates TRAP's ability to produce ROS, enabling the final breakdown of the bone matrix (Vääräniemi et al., 2004). It is possible that hypoxic-induced ROS accumulation may increase organic matrix breakdown. In addition to the normal function of ROS in osteoclasts, hypoxic microenvironments induce accumulation of mitochondrial ROS due to the

accumulation of reducing equivalents (Morten et al., 2013; Srinivasan & Avadhani, 2007). ROS produced by complex III of the ETC are of particular importance due to their involvement in mediating HIF-1α stabilisation. Prolonging HIF-1α stabilisation likely leads to further ROS accumulation and subsequent oxidative stress, activating osteoclasts (Bell, Emerling, Ricoult, & Guarente, 2011; Chandel et al., 1998). Furthermore, it has been noted that RANKL inhibits FoxO transcription and expression activity causing a decrease in the target gene for the antioxidant, Catalase, which scavenges hydrogen peroxide (H_2O_2; Bartell et al., 2014). Incidentally, mice deficient in FoxO transcription factors are shown to have increased levels of H_2O_2, bone resorption and subsequent reductions in trabecular bone (Bartell et al., 2014). This was further confirmed when mice that overexpressed mitochondrial catalase in osteoclasts displayed greater bone density (Bartell et al., 2014). Antioxidants appear capable of attenuating the ROS-induced osteoclast function, for example, in vivo administration of *N*-acetyl cysteine (NAC) has been shown to reduce osteoclast formation and subsequently reduce bone resorption (Lean et al., 2003).

7.2 | Osteoblasts

The overproduction of ROS appears to exert a similarly negative effect on osteoblasts. A. L. Liu, Zhang, Zhu, Liao, and Liu (2004) were amongst the first to report oxidative stress inhibited osteoblast differentiation, characterised largely by a reduction in ALP. Other differentiation markers were later noted to be inhibited under H_2O_2-induced oxidative stress, such as, phosphorylation of the transcription factor Runx2 and colony-forming unit-osteoprogenitor formation (Bai et al., 2004). It was confirmed to be the result of ROS production when metallothionein, an inhibitor of ROS production, restored osteoblast differentiation (A. L. Liu et al., 2004). ROS-induced inhibition was later found to be largely regulated through the extracellular-signal-regulated kinase (ERK) and NF-kB signalling pathways (Bai et al., 2004). Excessive ROS have since been shown to inhibit bone formation, specifically during mineralisation (Arai, Shibata, Pugdee, Abiko, & Ogata, 2007). In fact, exposure to nontoxic levels of H_2O_2 resulted in half the mineralisation of normal osteoblast function due to decreased ALP production (Arai et al., 2007). Although H_2O_2 may have mediated the response, it should be noted that other ROS may be responsible for the changes in bone metabolism (i.e., hydroxyl radical). In addition to the negative influences' ROS exert on osteoblast differentiation, they also modulate their lifespan. Glutaredoxin 5 (Grx5), a glutathione-dependent oxidoreductase, is highly expressed in bone and is involved in maintaining cellular redox homoeostasis (Linares, Xing, Govoni, Chen, & Mohan, 2009). Grx5 silencing resulted in apoptosis when exposed to H_2O_2, whereas overexpression of Grx5 in osteoblasts prevents ROS cell apoptosis (Linares et al., 2009). Similarly, the mitochondrial targeted drug, metformin, has been shown to reduce the hypoxic-induced oxidative stress and osteoblast apoptosis through suppression of cytochrome c release and cleavage of procaspase-9 and poly(ADP-ribose) polymerase (Lai et al., 2018). Oxidative stress may mediate osteoblast apoptosis through Wnt/B-catenin signalling (Manolagas & Almeida, 2007). The increased phosphorylation of the protein p66shc by oxidative stress may result in greater osteoblast apoptosis by redirecting B-catenin to FOXO transcription factors (Manolagas & Almeida, 2007). A summary of the afore-mentioned mechanisms can be found in Figure 3.

8 | ACIDOSIS AND BONE

Following tissue exposure to hypoxia, both respiratory and metabolic acidosis occur, shifting extracellular pH (Kingsley, Fournier, Chirgwin, & Guise, 2007; Lewis, Lee, Underwood, Harris, & Lewis, 1999; Raghunand, Gatenby, & Gillies, 2003; Swenson et al., 1991), a well-documented modulator of bone. While HIF-1α exerts both positive and negative effects on bone cells, acidosis is long known to stimulate unfavourable changes in bone (Goto, 1918). Early findings suggested that bone acted as a buffer to acidosis, but it was later found to directly inhibit and stimulate osteoblasts and osteoclasts, respectively (Bushinsky & Lechleider, 1987; Frick & Bushinsky, 1998). Several extensive reviews have summarised the distinct effects of acidosis on bone (Arnett, 2007, 2008, 2010; Yuan et al., 2016). Therefore, this review briefly discusses these as an associated factor of hypoxia.

8.1 | Osteoblasts

Osteoblasts are largely inhibited in acidic environments, specifically their mineralisation capacity (Brandao-Burch, Utting, Orriss, & Arnett, 2005). For example, osteoblast ALP activity is reduced eight-fold at pH 6.9 and its expression is similarly decreased (Brandao-Burch et al., 2005). Osteoblasts have a high sensitivity to pH, a 0.1 pH change is shown to reduce bone mineralisation three-fold (Brandao-Burch et al., 2005). Similar findings have been observed in human osteoblasts, whereby acidosis decreased matrix mineralisation (Disthabanchong, Radinahamed, Stitchantrakul, Hongeng, & Rajatanavin, 2007; Takeuchi, Hirukawa, & Togari, 2013). The decreased activity of ALP would explain the reduced mineralisation, as ALP is responsible for the hydrolysis of phosphate and supply of inorganic phosphate (Coleman, 2002). Others have noted significant increases in OPG in conjunction with decreases in ALP (Takeuchi et al., 2013). Treatment of cells with OPG in normal pH resulted in diminished matrix mineralisation, suggesting OPG may be involved in the acidic inhibition at physiological levels (Takeuchi et al., 2013). Despite low pH inhibiting mineralisation, collagen deposition remains stable and continues to be synthesised in vitro (Brandao-Burch et al., 2005). However, in the context of hypoxic-induced acidosis, this is unlikely to be the case due to reduced activity of PHD and lysyl oxidase enzymes, which are involved in the posttranslational modification of collagen (Myllyharju, 2003; Utting et al., 2006).

Chronic metabolic acidosis has been associated with decreased cell proliferation and altered differentiation. For example, Runx2

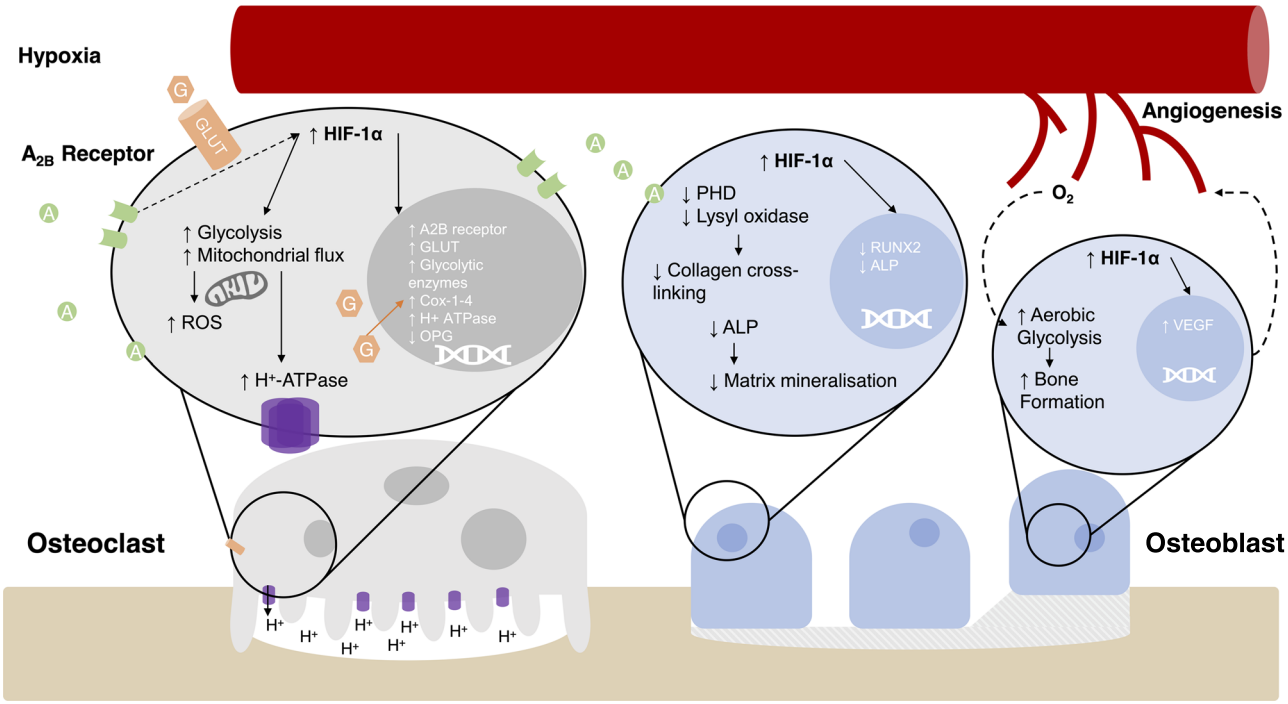

FIGURE 3 Effects of hypoxia/HIF-1 on osteoclast and osteoblast activity. Osteoclasts: Induction of HIF results in increased glycolytic activity and mitochondrial flux in osteoclasts. Increases in osteoclast metabolic activity results in greater H^+-ATPase activity and increased bone resorption. The hypoxic-induced metabolic flux results in ROS accumulation and increased TRAP activity. Stabilisation of the HIF protein leads to nucleus translocation and subsequent transcription responses. The increased concentration of glucose within hypoxic osteoclasts may also stimulate transcription of H^+ ATPase. Adenosine leakage from both osteoclasts and osteoblasts attaches to A_{2B} receptors on osteoclast, further stabilising HIF-1α in a positive feedback loop. Osteoblasts: (Left) Reduced oxygen availability results in PHD and lysyl oxidase inhibition resulting in impaired collagen cross-linking. ALP activity and transcription are also inhibited reducing calcification of the bone matrix. (Right) Proposed osteogenic–angiogenic signalling, whereby HIF-1a stabilisation increases transcription of the hypoxic response gene, VEGF, to stimulate angiogenesis and restore O_2 delivery and bone formation. A, adenosine; ALP, alkaline phosphatase; Cox, cyclooxygenase; OPG, osteoprotegrin; G, glucose; HIF, hypoxic-inducible factor; PHD, prolyl hydroxylase domains; ROS, reactive oxygen species; RUNX2, Runt-related transcription factor 2; VEGF, vascular endothelial growth factor

expression was greater in acidosis while levels of osterix, a transcription factor for osteoblast differentiation, were suppressed resulting in altered differentiation of osteoblasts (Disthabanchong et al., 2007). Furthermore, cell viability is decreased in low pH conditions until eventually triggering osteoblast apoptosis at pH 6.0 (Zhang et al., 2017). As pH declines, increased levels of P62, the primary substrate of autophagy and LC3-II expression, a protein marker of autophagy, have been demonstrated in osteoblasts. Of note, suppression of autophagy increases osteoblast apoptosis following acidic exposure, suggesting that autophagy may act as a survival mechanism in acid conditions (Zhang et al., 2017). Ultimately, the evidence suggests that osteoblast function appears to be significantly hindered in acidic environments.

8.2 | Osteoclasts

Osteoclasts are typically shown to be almost inactive at normal physiological pH (~7.4). However, when pH is reduced below 7.6, significant increases in osteoclast pit formation are observed before plateauing at a pH of 6.8 (Arnett & Dempster, 1986). In primary human osteoclasts, a change <0.1 unit pH doubles their resorptive activity (Arnett, 2008; Arnett & Spowage, 1996). Regardless of exposure length, osteoclasts maintain their resorptive capacity and continue to increase their resorption capacity in response to small pH changes (Arnett, 2010). Acidosis is thought to play three distinct regulatory roles in osteoclasts:

(a) Differentiation: Acidosis-induced osteoclast formation has been reported to target late preosteoclast differentiation (Kato & Matsushita, 2014; Kato & Morita, 2011). Cultures of bone marrow cells in acidic compared to normal pH media, promoted formation of large osteoclasts via late phase preosteoclast differentiation to osteoclasts (Kato & Morita, 2011). Subsequently, osteoclasts formed under acidic conditions display greater resorption capacity than those matured at higher pH (Kato & Morita, 2011). However, mRNA levels of NFATc1 and DC-STAMP, molecules intimately involved in cell differentiation and fusion, revealed no significant differences between cells treated with pH 6.8 or 7.4. Kato and Matsushita (2014) suggested that acid-induced ROS

(Riemann et al., 2011) may be responsible for osteoclastic differentiation. The use of the antioxidant NAC has been shown to inhibit osteoclast formation suggesting that osteoclasts are, somewhat, dependent on ROS (Kato & Matsushita, 2014; Lean et al., 2003). It is still uncertain how acidosis stimulates osteoclast differentiation and future investigations are needed to clarify the exact mechanism. However, acidosis as a result of hypoxia may exert a cumulative effect in hypoxia-induced osteoclast differentiation. As previously noted, hypoxia is a known stimulant of ROS, beyond ROS production via acidosis per se. Therefore, it may be that hypoxia is capable of large increases in osteoclast numbers due to accumulative ROS generation from differing sources.

(b) Activation: Acid activation of osteoclasts has been reported extensively (Arnett & Dempster, 1986; Frick & Bushinsky, 2003; Indo et al., 2013; Yuan et al., 2016). The excessive level of resorption has been demonstrated by complete perforation of neonatal mouse calavaria samples (Meghji, Morrison, Henderson, & Arnett, 2001). Furthermore, activation of osteoclasts allows for other factors to exert further resorptive stimuli (Arnett, 2008; Arnett & Dempster, 1986; Morrison et al., 1998). Evidence suggests that acidosis may activate osteoclast activity through both direct and indirect methods. Acidosis has been shown to increase the expression of key resorption tools such as carbonic anhydrase II (Biskobing & Fan, 2000), cathepsin K and TRAP (Brandao-Burch et al., 2003; Muzylak, Arnett, Price, & Horton, 2007) while rapidly increasing intracellular Ca^{2+} (Komarova, Pereverzev, Shum, Sims, & Dixon, 2005). The increased intracellular Ca^{2+} causes further activation of NFATc1 and subsequent elevations in resorption. Others have found that osteoblasts produce prostaglandin E2 in response to acidosis stimulating osteoclastic bone resorption (Bushinsky, Parker, Alexander, & Krieger, 2001; Frick & Bushinsky, 2003; Krieger, Parker, Alexander, & Bushinsky, 2000). Furthermore, acidosis increases adhesion and migration of osteoclasts allowing for greater bone resorption (Ahn, Kim, Lee, Kim, & Jeong, 2012).

(c) Survival: The effect of acid-induced NFATc1 on osteoclasts has been shown to be two-fold. Not only have reports noted its effect on osteoclast activation, but it is also involved in prolonging osteoclast life. Alongside RANKL, NFATc1 is an inhibitor of osteoclast apoptosis (Yuan et al., 2016). As stated, increased intracellular Ca^{2+} activates NFATc1, but it has also been shown to activate the mitogen-activated protein kinase (MAPK) pathway, which is also important for osteoclast survival (Komarova et al., 2005; Yuan et al., 2016).

9 | ERYTHROPOIETIN AND BONE

The immediate response to hypoxia is the result of HIF activation to prolong survival. As part of this survival response, HIF targets the hypoxic response element for transcription of 90–100 genes (Choudhry & Harris, 2018). One of particular importance is erythropoietin (EPO), a hormone for stimulating red blood cell production. While hepatocytes remain the primary source of EPO expression, other cells located in the brain, liver and bone are capable of stimulating EPO production (Jelkmann, 2011). EPO expression is predominantly controlled by HIF-2 stabilisation and subsequent transcription (Warnecke et al., 2004). Following tissue hypoxia, circulating serum EPO can increase by 1,000-fold (Ebert & Bunn, 1993). The subsequent interaction of EPO to its receptor EPO (EPO-R) in erythroid progenitor cells, located in the bone marrow, signals Janus kinase 2 (JAK2), which is a transducer and activator of transcription 3 (STAT3) and STAT5 pathways for erythropoiesis (Jelkmann, 2011). Several clinical observations have observed correlations between EPO-related diseases such as polycythaemia and impaired bone health (Farmer, Horváth-Puhó, Vestergaard, Hermann, & Frederiksen, 2013; Oikonomidou et al., 2016). This has raised concerns about recombinant use of EPO for both clinical and ergogenic purposes (i.e., for sports and exercise performance). However, further experimental research is needed to determine causality.

Like HIF-1α, EPO has been found to stimulate osteogenesis. Using a femoral fracture repair murine model, exogenous administration of EPO has been shown to improve both biomechanical and volumetric measures of bone (Holstein et al., 2011; L. Wan et al., 2014). It is suggested that osteoblastic expression of EPO receptors (EPO-R) and subsequent interaction with mammalian target of rapamycin (mTOR), JAK2 and PI3K signalling pathways, produce a osteogenic response (J. Kim et al., 2012; Rölfing et al., 2014). Exogenous EPO treatment has also been reported to be associated with increased angiogenesis, which alluded to previously, is key to the osteogenic–angiogenic coupling response (Holstein et al., 2011; Rölfing et al., 2012; L. Wan et al., 2014). It has also been suggested that EPO plays an important role in angiogenesis, as deletion of EPO or EPO-R from endothelial cells leads to improper vascularisation during development (Eggold & Rankin, 2018; Kertesz, Wu, Chen, Sucov, & Wu, 2004). The role of EPO in the angiogenic response is reportedly dependent on normal osteoclastogenesis. When blocked with OPG or bisphosphonates, the reported EPO-induced angiogenesis was largely inhibited (Sun, Jung, Shiozawa, Taichman, & Krebsbach, 2012). Others have suggested that EPO stimulates osteoblastic differentiation through direct interaction with mesenchymal and hematopoietic stem cells, or indirect stimulation of hematopoietic stem cell production via bone morphogenetic protein (J. Kim et al., 2012; Rölfing et al., 2014; Shiozawa et al., 2010).

Though several studies report an osteogenic effect of EPO, the dosages utilised would be considered supra-physiological. Hiram-Bab et al. (2017) note that in vitro doses between 10 and 100 U/ml (Guo et al., 2014; J. Kim et al., 2012; C. Li et al., 2015; Rölfing et al., 2014) sufficiently invoke osteogenesis whereas dosages 1–10 U/ml do not (Guo et al., 2014; Hiram-Bab et al., 2015; Rölfing et al., 2014). This is an important consideration as human endogenous EPO production ranges between 6 and 32 mU/ml (Jelkmann, 2011), ultimately questioning the physiological relevance of some findings. Considering this, it is unlikely that endogenous production through hypoxic exposure would stimulate any osteogenic effect. Nonetheless, higher exogenous doses are indeed used in clinical populations and are therefore important.

Beyond the osteogenic response noted previously, several observations have found paradoxical increases in bone resorption, decreases in bone formation and bone volume in both exogenously treated mice and in EPO overexpressing transgenic mice (Deshet-Unger et al., 2016; Hiram-Bab et al., 2015; Oikonomidou et al., 2016; Rauner et al., 2016). More importantly, low physiological doses of EPO, similar to that of the increase in EPO following hypoxic exposure (10 mU/ml; Mackenzie, Watt, & Maxell, 2008; Mounier et al., 2009; Turner et al., 2017), have been shown to stimulate in vitro osteoclastogenesis (Hiram-Bab et al., 2017). It is suggested that EPO directly stimulates EPO-R activation of the JaK2 and PI3K pathways, subsequently stimulating osteoclastogenic activity (Hiram-Bab et al., 2015, 2017; Rauner et al., 2016). Hiram-Bab et al. (2015) noted that EPO stimulates differentiation of pre-osteoclasts to mature osteoclasts, subsequently increasing resorption. Interestingly, osteoclast differentiation decreased EPO-R transcription suggesting that EPO-R expression is restricted to pre-osteoclasts (Hiram-Bab et al., 2015). Though it is apparent that osteoclastogenesis is a distinct effect of EPO, it is unclear whether EPO stimulates osteoclast activity independent of increased osteoclast number (Hiram-Bab et al., 2015). Shiozawa et al. (2010) have previously suggested that EPO inhibits osteoclast activity, but this has since been refuted as bone resorption and tartrate-resistant acid phosphatase^{+} (TRAP^{+}) area are unaffected in cultures treated with EPO (Hiram-Bab et al., 2015). However, current evidence does not suggest that osteoclast activity is stimulated following increases in EPO.

Osteoblast-specific cultures have demonstrated that physiological ranges of EPO inhibit mineralisation while supra-physiological dosages increased formation in a dose response manner (Deshet-Unger et al., 2016; Hiram-Bab et al., 2015, 2017; Rauner et al., 2016). Differentiation of bone marrow stromal cells with a low EPO dosage (1 mU/ml) inhibited expression of Runx2, osteocalcin and ALP, whereas a higher dose had no effect (25 mU/ml; Rauner et al., 2016). Silencing of EPO-R using siRNA ablated the inhibition of bone formation suggesting EPO-R signalling is responsible for osteoblast inhibition (Rauner et al., 2016). Others have noted increased expression of fibroblast growth factor 23 (FGF23) in bone marrow haematopoietic stem cells in response to EPO, subsequently increasing serum FGF23 and decreasing phosphate (Chang et al., 2008). It is suggested that the increased levels of EPO may inhibit bone mineralisation through the bone-kidney-parathyroid gland axis (Sapir-Koren & Livshits, 2011; H. Wang et al., 2008).

Interestingly, Rauner et al. (2016) found that loss of the PHD2 enzyme in EPO producing cells subsequently led to impaired bone density, attributed to decreased osteoblast function; whereas measures of osteoclast function revealed no changed. Loss of PHD2 in the osteoblastic lineage resulted in significantly greater femoral and lumbar bone density which was associated with decreased osteoclast number and surface (Rauner et al., 2016). Deletion of PHD2 in haematopoietic cells however did not result in increased bone resorption or decreased bone mass, contradictory to other findings (Hulley et al., 2017; Wu et al., 2015). Rauner et al. (2016) suggests that osteoblast inhibition in mice with conditional loss of PHD2 is independent of any intrinsic effect. The discrepancy between the osteogenic and resorption effect of EPO is poorly understood but may be the result of large differences in dosages of EPO and in vitro models used. Nonetheless, EPO is a downstream target of the HIF pathway and clearly plays a role in modulating bone cell activity, at least in animal models. In conjunction with HIF-mediated changes, exposure to hypoxia may lead to further alteration in bone via increases EPO.

10 | IN VIVO HYPOXIA

Hitherto, this review has mostly examined the molecular response of bone cells to hypoxia. However, it is also pertinent to inspect the responses from the available in vivo studies (for a more comprehensive overview, please see the work of Camacho-Cardenosa, Camacho-Cardenosa, Timón, et al. (2019). Like the other experimental approaches considered in this review, it has been suggested that different types of in vivo hypoxic exposure may elicit different responses. For instance, Camacho-Cardenosa, Camacho-Cardenosa, Timón, et al. (2019) systematically reviewed available in vivo studies, concluding that the different types of hypoxic exposure sustained: cyclic and intermittent, may be responsible for some of the observed changes in bone.

Long-term sustained exposure has been reported to reduce several indices of bone health (Basu et al., 2013, 2014; O'Brien et al., 2018; W. Wang et al., 2017) whereas shorter term sustained exposure appears less noticeable (Rittweger et al., 2016). Bone's varied response to hypoxia may be a function of the oxygen concentration it receives. For instance, Rittweger et al. (2016) simulated 4,000 m altitude during bed rest in humans for 21 days. Basu et al. (2013) monitored members of the Indian army exposed to real altitudes of 5,400 to 6,700 m over 4 months and W. Wang et al. (2017) exposed rats to a simulated ~6,000 m for 21 days. The difference in altitude, and outcomes, suggests a possible hypoxic threshold, which may account for the lack of effect during Rittweger et al. (2016) research. Considering sea level FiO_2 is 20.9%, and that bone O_2 is thought to rest between 6.6% and 8.6%, then at ~4,000 m (~12.8% O_2; 38.7% decrease from sea level) bone oxygen delivery would be ~4–5.3%. Whereas at 6,000 m, the effective oxygen reaching bone would be ~3.1–4%. Based upon the findings of Arnett et al. (2003), 6,000 m would be closer to maximally stimulating osteoclast resorption activity (i.e., 2% O_2) than 4,000 m. While this appears a small difference, it has been noted that 2% O_2 stimulates osteoclast resorption approximately twice that of 5% O_2 exposure (Arnett et al., 2003). Although it is important to note the differing oxygen concentrations in such studies; it is unlikely that bone O_2 was stable during the course of each study due to variations in experimental conditions that is bed rest versus field research.

Cyclic exposure appears to produce varying responses. One study has reported improvements in structural measures of bone following 5 weeks of exposure of 4,500 m, lasting 5 hr a day for 5 days a week (Guner et al., 2013). Yet, others have noted that less severe

TABLE 1 Summary of the effects of hypoxia on osteoblasts and osteoclasts

Cell	Stimulus	Effect on bone remodelling	Mechanisms
Osteoblasts	Hypoxia/HIF-1α	• Increased bone formation • Delayed growth and differentiation • Suppressed osteoblastogenesis • Decreased mineralisation and collagen production	• Osteogenic–angiogenic coupling • Reduced Runx2 expression • PI3K/Akt inhibition • Reduced ALP expression and activity • Inhibition of collagen cross-linking enzymes
	Glycolysis:	• Increased bone formation	• Increased Runx2 and GLUT1 expression
	ROS	• Inhibited differentiation • Decreased mineralisation • Increased apoptosis	• Decreased ALP, Runx2 and colony-forming unit osteoprogenitor • ERK and NF-kB stimulation • Increased p66sch and B-catenin redirection
	Acidosis	• Decreased differentiation • Reduced mineralisation • Decreased cell proliferation • Decreased cell viability	• Inhibition of mRNA Runx2 and osterix • Reduced ALP expression • Reduced OPG • Increased P62 and LC3-II
Osteoclasts	Hypoxia/HIF-1α	• Increase resorption • Increased differentiation • Osteoclastogenesis	• PHD2 induction of HIF-1α • PHD3 induction of HIF-1α • Suppressed OPG production
	Glycolysis	• Increased resorption	• Increased A-subunit of vacuolar H+ ATPase and subsequent H+ production
	ROS	• Increased resorption	• Increased collagen breakdown
	Acidosis	• Increased differentiation • Increased resorption • Increased survival	• Increased expression of carbonic anhydrase II, cathepsin K and TRAP • Increases in prostaglandin E2 • Increases in NFATc1 and MAPK signalling

Abbreviations: ALP, alkaline phosphatase; ERK, extracellular-signal-regulated kinase; GLUT1, glucose transporter 1; HIF, hypoxic-inducible factor; MAPK, mitogen-activated protein kinase; NFATc1, nuclear factor-activated T cells c1; NF-kB, nuclear factor-kB; OPG, osteoprotegerin; PHD2, prolyl hydroxylase domain 2; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; Runx2, runt-related transcription factor 2.

exposures are unable to yield similar responses (Camacho-Cardenosa, Camacho-Cardenosa, Burtscher, et al., 2019; Martínez-Guardado et al., 2019; Ramos-Campo, Rubio Arias, & Jimenez Diaz, 2015). Similarly, no differences in structural measures of bone have been shown when comparing healthy rats exposed to 3,000 to 5,000 m 4 hr/day for 14 days when compared to normoxic controls. Interestingly, when rats were OVX, hypoxia was shown to intensify bone loss (Xian et al., 2016). The variations in responses to cyclic hypoxic exposures may be explained by the variance in protocols that is the hypoxic dose and exposure time, but this is yet to be fully understood. Intermittent hypoxia, like that of obstructive sleep apnoea syndrome (Swanson et al., 2015), also has varying effects on bone. Some studies document reductions in bone health (Terzi & Yılmaz, 2016; Tomiyama et al., 2008) whereas others present greater BMD preservation in older individuals (Sforza, Thomas, Barthelemy, Collet, & Roche, 2013). It appears apparent that hypoxia is capable of manipulating bone in vivo, but that it may, crucially, be dependent on the exposure level, time and frequency. Future research should strive to understand the time response to hypoxic stimuli both constant and cyclic.

11 | CONCLUSION

Reduced oxygen availability is accompanied by the stimulation of hypoxic pathways, glycolysis, ROS, acidosis and downstream effects (Arnett, 2010; Jelkmann, 2011; Zepeda et al., 2013). This hypoxia appears to be an important mediator of bone metabolism and may be associated with poor bone health. While hypoxia generates a more consistent bone response in vitro; the response to hypoxic mimicking agents and gene therapy appear more varied. Generally, hypoxic signalling appears capable of providing both positive and negative influences on bone cells (see Table 1). An antagonistic relationship may exist to provide balance in bone metabolism under conditions of hypoxia; yet, when assessing in vivo hypoxic exposure this is not consistently found within the available scientific literature, possibly owing to variations in hypoxic dose and exposure type. Hypoxia clearly presents itself as a regulator of bone cell function and potentially offers therapeutic options but may also have negative effects for bone health; however, there is currently a lack of experimental human research investigating bone's response to hypoxic stimuli. Therefore, further research is warranted, particularly aimed at comparing responses to differing hypoxic protocols. The effects of hypoxia in pathological conditions is of utmost importance as many of these conditions appear to be related to impairments in BMD (Ramachandran et al., 2016; Terzi & Yılmaz, 2016; Valderrábano et al., 2017). It is also important to consider the implications of hypoxia on individuals exposed to environmental hypoxia. For example, mountaineers, athletes attending high altitude camps, astronauts, pilots and native highlanders may be at risk of compromised bone health (Tanaka et al., 1992; Tando et al., 2016; Terzi & Yılmaz, 2016; W. Wang et al., 2017). However, it is important to recognise that all in vivo findings to date, do not support the notion that

hypoxia poses a risk to bone. Furthermore, it is interesting to consider exercise as an endogenous hypoxic stimulus. Many studies note increases in bone resorption markers following acute exercise (Barry & Kohrt, 2007; Sale et al., 2015; Scott et al., 2011). Such increases in bone turnover may be associated with the metabolic demands of exercise and the stabilisation of HIF (Baker & Parise, 2016; Milkiewicz et al., 2007; Vogt et al., 2001). Further research is warranted on such populations to determine whether exercise or environmental exposure pose a risk or benefit to bone health, both acutely and chronically.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

S. H.: conceptualisation, writing—original draft, review, editing, and visualisation. C. M., A. M. and S. M.: review, editing, and supervision. All authors read and approved the final submitted manuscript.

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